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(71) Applicant: DEPARTEMENT CENTRAL DE FRACTIONNEMENT DE LA CROIX-ROUGE S.C.R.L. [BE/BE]; Avenue de Tyras 109, B-1120 Brussels (BE).

(71) Applicants and
(72) Inventors: LAUB, Ruth [BE/BE]; Avenue Besme 6, B-1190 Brussels (BE). DI GIAMBATTISTA, Mario [IT/BE]; Rue Plouchart 24, B-7090 Braine-le-Comte (BE).

(74) Agents: VAN MALDEREN, Eric et al.; Office Van Malderen, Place Reine Fabiola 6/1, B-1083 Brussels (BE).

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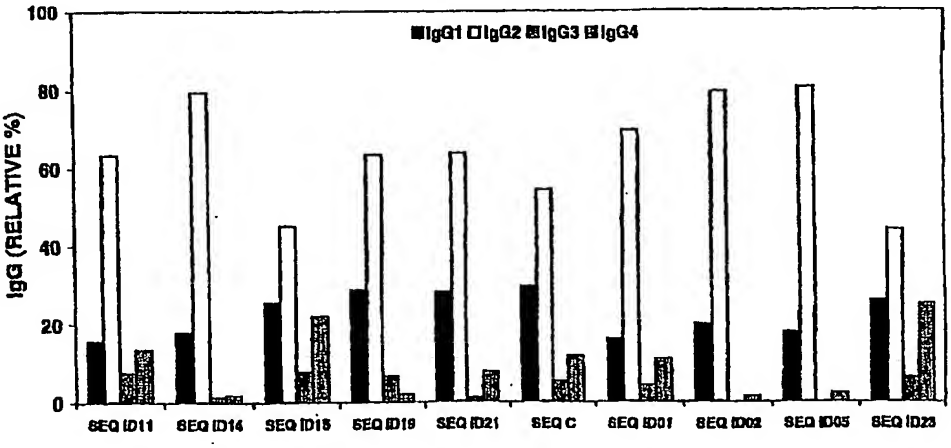
(54) Title: ANTIGENIC EPITOPES OF FACTOR VIII, INHIBITORS DIRECTED AGAINST SAID EPITOPES AND USE THEREOF



(57) Abstract: The present invention is related to antigenic polypeptide sequence (epitope of factor VIII) to the inhibitors which are directed against these sequence and to anti-inhibitors which are directed against said inhibitors. The present invention is also related to pharmaceutical composition and to a diagnostic device comprising at least one of the above mentioned molecules.

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ANTIGENIC EPITOPES OF FACTOR VIII, INHIBITORS DIRECTED AGAINST SAID EPITOPES AND USE THEREOF

Subject of the invention

[0001] The present invention relates to the antigenic polypeptide sequences (epitopes) of factor VIII to the anti-FVIII inhibitors which are directed against these sequences and to anti-inhibitors which are directed against said anti-FVIII inhibitors.

[0002] The present invention also relates to a pharmaceutical composition and to a diagnostic device comprising at least one of the above mentioned molecules.

Technical background underlying the invention

[0003] FVIII is a large multi-domain protein of 2,332 amino acids made up of three structural domains, A, B and C which are arranged in the order A1:a1:A2:a2:B:a3:A3:C1:C2. The A domains possess more than 40% homology and are also homologous to ceruloplasmin (for recent review, see Pratt (2000) and Saenko (1999)). 30% homology also exists between the A domains of factor V and FVIII. The C domain occurs twice and is reported to be able to bind glyco-conjugates and phospholipids having a net negative charge. It exhibits homology with lectins which are able to bind to negatively charged phospholipids. The platelet attachment site has been located in this region (C2 domain) (Foster et al., (1990)).

[0004] These antigenic determinants consist of fragments 351 - 365 (A1 domain - heavy chain), 713 - 740 (A2 domain), 1670 - 1684 (A3 domain - light chain) (NH_2 end of the light chain) or else 2303 - 2332 (C2 domain - light chain) (Foster C, (1990)), fragments 701 - 750, 1663 - 1689, 330 - 472, 1694 - 1782 (EP-0 202 853), 322 - 740 and 2170 - 2322.

[0005] The U.S patent 5,744,446 describes an hybrid human/animal Factor VIII having a sequence of amino acids selected from the group of the A2 domain fragments 373-540, 373-508, 445-508, 484-508, 404-508, 489-508 and 484-489, with corresponding sequences of porcine or murine Factor VIII, said hybrid being used for the treatment of Factor VIII deficiencies.

[0006] The antibodies which recognize these various sites interfere, with the activation of FVIII, the binding of vWf, FIXa, FXa, APC or phospholipids. The specific antibody response to FVIII vary considerably among individuals, and epitopes for inhibitor antibodies have to be determined for all FVIII domains (see for recent review Scandella, 2000; Lollar, 2000).

[0007] Other antibodies, which do not inhibit standard activity tests in vitro, can exert an influence on the behavior of FVIII with the other constituents of the coagulation cascade while attaching themselves to sites in the molecule which are at a substantial distance from the active sites. These antibodies, can interfere with the natural state of folding of FVIII by altering some of its properties.

[0008] Emergence of alloantibodies (inhibitors) that neutralize infused FVIII activity may seriously complicate FVIII replacement therapy. Reported inhibitor incidence rates in hemophiliacs vary considerably. They range around 6-35% (Vermylen et al, 1998). Candidates for genetic

predispositions such as large deletions and intron 22 inversion have been found associated with a high incidence of inhibitors and genes that are involved in the immune response as genes MHC class I and class II (Tuddenham and McVey, 1998). Repeat switching from one FVIII product to another and the possibility that some FVIII concentrates are more immunogenic may also explain the appearance of inhibitors (Vermylen et al, 1998). Different methods of preparing FVIII could exert an influence on its structure, its physicochemical properties or its natural micro-environment; Laub et al. (1999); Raut et al. (1998)). Clinically relevant anti-FVIII autoantibodies are rare in non-hemophilic patients (annual frequency in the population: 1-5/10^6) (Morrisson and Ludlam) (1995). They are associated with a number of autoimmune diseases and are often characterized by life-threatening hemorrhage. On the other hand, anti-FVIII antibodies have also been described in healthy subjects (Algiman et al, 1992; Moreau et al, 2000), without any apparent effect on the subjects' levels of circulating FVIII.

[0009] Self proteins or derived peptides may elicit an immune response if presented to CD4 T cells at inflammatory sites by professional antigen presenting cells. Using pools of overlapping synthetic peptides spanning the sequences of individual FVIII domains, Reding et al. (2000) showed reactive $CD4^+$ to FVIII in healthy subjects and hemophilia patients. Several FVIII domains were recognized: A3 domain was recognized more strongly and frequently and each domain forms several epitopes.

[0010] Techniques such as western blotting, immunoprecipitation, and enzyme-linked immunosorbent assays (ELISAs), using well-defined FVIII proteolytic fragments, a large recombinant peptide library, or synthetic peptide arrays, have been used to map different FVIII-inhibitor

binding sites located mainly in the A2 and C2 domains. However, none of these techniques has made it possible to build a model for identification of inhibitor and non-inhibitor epitopes. Only a few epitopes have been mapped to discrete sequences (<20 amino-acid residues). To solve this problem, Palmer et al (1997) synthesized 96 undecamer peptides (11 amino-acid residues) representing 80% of the complete residue sequence of FVIII. They succeeded in determining the epitope specificity of 9 patients' inhibitory antibodies. Other useful techniques are analysis of FVIII gene mutations and their effects on the FVIII molecule as well as phage display technology (van den Brink et al, 2000). All these methodologies, however, are time consuming, rather costly, and largely dependent on patient availability. Certain areas of the FVIII molecule may be "hot spots" containing commonly recognized clusters of inhibitor epitopes, e.g., regions in the A2 domain, A3 domain, and C2 domain. The reason for these "hot spots" in generating an inhibitor response remains poorly understood (Reisner et al, 1995).

[0011] Currently, a predominant notion among hemophilic patients, clinicians and "fractionators" is that of having available a purified FVIII which is devoid of all pathogenic plasma contaminants and secondary effects.

[0012] Different animal models could be used as hemophilia dogs, scid mice, hemophilia mice ... but until now, no satisfactory experimental model exists which makes it possible to forecast the immunogenicity or the immuno-modulatory effect of the FVIII preparations, or the susceptibility of the host, before they have been administered clinically.

[0013] Patients who develop an anti-FVIII immune response find themselves in a serious situation which

necessitates the use of severe, aggressive and excessively expensive measures.

[0014] One of the frequently treatment, is the induction of immune tolerance by administration of very high doses of FVIII (150 IU/kg twice a day) in association or not with prothrombin complex concentrates and is assigned as "Bonn Protocol". Treatment options are also to by-pass the FVIII inhibitor activity by use of PCC (preferably an activated PCC [APCC]) or FVIIa. Specific antibodies as consequence of the infusion of these alternative agents could be produced, impairing the treatment. As an alternative agent porcine FVIII may be used to achieve haemostasis in patients with antibodies that do not substantially crossreact with porcine FVIII before or during the treatment (Lollar, 2000).

[0015] A potential alternative approach to inhibit the production of inhibitors is blockade of the T cell/B cell collaboration mediated by through receptor ligand binding signal events (Ewenstein et al, 2000). Preliminary clinical trials were performed using a humanized mouse monoclonal antibody to human T cell CD40 ligand (CD 154).

[0016] A profitable strategy for reducing the level of inhibitors has consisted in subjecting patients to an extracorporeal circulation to enable solid-phase absorption of the total IgG.

[0017] The immunoabsorbant could be sepharose-bound staphylococcal protein A or sepharose-bound polyclonal sheep antibodies to total human immunoglobulin (Knobf and Derfler, 1999). The foreign proteins (protein A, sheep anti-human Ig) could leak from the column and triggered the immune system of the recipient; moreover problems could raised as sanitisation (ICH Topic Q5A, Directive 92/79/EC).

[0018] The infusion of polyvalent intravenous immunoglobulins (IVIG), where appropriate combined with an immunosuppressive treatment, has been found to be relatively effective, although the reason for this effectiveness is still not fully established. Various hypotheses involving feed-back inhibition of IgG synthesis, stimulation of IgG clearance or activation of T suppressor cells have been advanced. An interesting explanation is that these commercial intravenous immunoglobulins might contain antibodies which are able to react with the variable parts (idiotypes) of the anti-FVIII antibodies and neutralize these antibodies (Dietrich et al. (1992)).

[0019] Unfortunately, none of these approaches has been found to be satisfactory in terms of safety, efficacy, efficiency and cost.

[0020] The state of the art in epitope structure prediction was limited given to the fact that non-continuous amino acid residues seem to constitute most important epitope and that the dynamics of binding is often not integrated into the epitope prediction equation making epitope structure prediction a complex four-dimensional problem (Van Regenmortel, Methods: A companion to Methods in Enzymology, 9, page 465-472, 1996).

[0021] According to the author, most of the antibodies raised against intact proteins do not react with any peptide fragment derived from the parent protein indicating that such antibodies are directed to discontinuous epitopes (conformational epitopes).

[0022] This author states also that low success rate of antigenic prediction is due to the fact that predictions concerns only continuous epitopes and it is unrealistic to reduce the complexity of epitopes that always possess conformational features to one dimensional linear peptide model.

[0023] Similarly, Palmer et al. (1997) using synthetic peptide arrays to identify novel Factor VIII inhibitor epitopes note that each patient pattern of anti-factor VIII antibody reactivity appears to be polyclonal, directed against multiple sites located within the amino and carboxyl terminus of the protein and seems to be unique for each plasma investigated (see also above).
Moreover, this author notes that it is difficult to predict the importance that any given antibody: epitope interaction may have on Factor VIII coagulation activity based on the results of synthetic peptide assays alone (due to the uncomplete understanding of the relationship between structure and function of different factor VIII domains and the possibility that both inhibitor and non-inhibitory antibodies may be present in a patient's plasma.

[0024] Therefore, the documents of the state of the art do not suggest to identify antigenic linear peptides upon a macro-molecule (such as Factor VIII) and that linear epitopes could be used for the diagnostic and/or the therapy of immune disorders induced by inhibitors directed against Factor VIII.

[0025] The international patent application WO96/02572 describes antigenic fragment and epitope sequences of factor VIII and inhibitors directed against some of these sequences.

Aims of the invention

[0026] The present invention aims to obtain new antigenic epitopes of factor VIII in order to improve the diagnosis and/or the therapy (including the prevention) of immune disorders (in particular those induced by inhibitors of FVIII, especially inhibitors of the binding of FVIII to the von Willebrand factor (vWf), to the FIX and/or to membrane phospholipids (PL)), said epitopes allowing a

screening between non-inhibitory and inhibitory anti-FVIII allo- or auto-antibodies (allo- or auto-immunoglobulins).

[0027] Another aim of the invention is to obtain inhibitors which exhibit an immunoaffinity with these antigenic epitopes, as well as to obtain anti-inhibitors, in particular antibodies or (T)cell receptors, which are directed against the abovementioned said inhibitors and whose purpose is to improve the diagnosis and/or therapy (or prevention) of immune disorders.

[0028] A further aim of the invention is to obtain said molecules at high purity, in industrial level, without contaminants (viruses, prions,...) and according to the GMP practices in the field of therapy and diagnostics (ICH topic QSA, Directive 92/79/EC, etc.).

Summary of the invention

[0029] The present invention relates to the antigenic polypeptide sequences (epitopes) of factor VIII whose complete sequence is described by Verhar et al. (1984) and which provides the reference for the amino-acids numerotation of the complete factor VIII sequence.

[0030] The "complete polypeptide sequence of factor VIII" is understood to be the natural human or animal sequence, which may be glycosylated and which has been obtained by purification from pools of plasma, in particular cryoprecipitate, by synthesis and/or by genetic manipulation (sequence from which portions which are not involved in the mechanism of blood coagulation may have been deleted) of factor VIII.

[0031] The present invention relates, in particular, to an antigenic epitope sequence of factor VIII which is selected from the group consisting of :

- the epitope comprised between serine 2018 and histidine 2031 inclusive, defined by the following sequence:

SEQ ID No:10:

Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His
1 5 10

- the epitope tyrosine 555 to glutamine 565 inclusive, defined by the following sequence:

SEQ ID No:17:

Tyr Lys Glu Ser Val Asp Gly Arg Gly Asn Gln
1 5 10

- the epitope leucine 730 to serine 741 inclusive, defined by the following sequence (P4):

SEQ ID No:20:

Leu Leu Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser
1 5 10

possibly deleted from the terminal amino acid serine (P4) and/or the first amino acid leucine

- the epitope serine 817 to serine 830 inclusive, defined by the following sequence (P5):

SEQ ID No:21:

Ser Asp Asp Pro Ser Gly Ala Ile Asp Ser Asn Asn Ser
1 5 10

- the epitope asparagine 2128 to asparagine 2138 inclusive, defined by the following sequence:

SEQ ID No:24:

Asn Val Asp Ser Ser Gly Ile Lys His Asn
1 5 10

- the epitope serine 2204 to glutamine 2222 inclusive, defined by the following sequence (P12):

SEQ ID No:27:

Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp
1 5 10 15
Arg Pro Gln

- the epitope isoleucine 2262 to glutamine 2270 inclusive, defined by the following sequence:

SEQ ID No:30:

Ile Ser Ser Ser Gln Asp Gly His Gln
1 5

- the epitope leucine 2273 to serine 2289 inclusive, defined by the following sequence (P14):

SEQ ID No:31:

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
1 5 10 15
Ser

- the epitope proline 2292 to tyrosine 2305 inclusive, defined by the following sequence (P15):

SEQ ID No:32:

Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr
1 5 10

possibly deleted from one or more amino acids of the terminal tripeptide Thr-Arg-Tyr involved in the phospholipid von Willebrand factor binding site

- the epitope glutamic acid 2322 to tyrosine 2332 inclusive, defined by the following sequence (P16):

SEQ ID No:33:

Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
1 5 10

[0032] The invention also relates to the major parts of the said epitopes. Said epitopes can be deleted from one or more terminal amino acids, preferably from one, two or three amino acids, or can be replaced by one or more amino acids that present the same characteristic of hydrophilicity, flexibility and accessibility.

[0033] It is also known that some of the epitopes according to the invention are comprised in major determinants of human inhibitors epitopes or several factors binding sites or binding sites of known monoclonal antibodies, especially the portion C2 that is known to be the binding site of the monoclonal antibody Mas531P or the binding site ESH8 as well as phospholipids, Factor Xa or the von Willebrand factor binding site. However, the specific epitopes according to the invention or their major

parts are preferred selected portions of said binding sites or may include a possible overlapping with said binding sites.

[0034] These epitopes sequences are particularly advantageously characterized by high hydrophilicity, which has been defined by Parker and Hodges (1986), considerable flexibility, which has been defined by Karplus and Schultz (1985) and considerable accessibility, which has been defined by Janin (1979).

[0035] These epitopes are, in particular, exposed on the surface of the factor VIII protein and exhibit pronounced antigenic and immunogenic characteristics.

[0036] Another aspect of the present invention is related to a modified (recombinant or transgenic) FVIII, possibly obtained by genetic engineering, and deleted from one or more of the above-identified epitopes or major parts of said epitopes.

[0037] Advantageously, said FVIII still allows the binding of coagulation factor(s), but will be less immunogenic and will not induce or induce less the formation of inhibitors directed against said modified FVIII or natural FVIII.

[0038] Advantageously, said epitopes are also independently immunogenic (that is to say they are immunogenic even without being complexed with a protein of large size such as BSA, KLH, haemocyanin, etc.), and preferably exhibit an immunoaffinity within inhibitors of factor VIII, such as anti-factor VIII antibodies, and/or exhibit an immunoaffinity for the receptors of the T lymphocytes and possibly B lymphocytes.

[0039] These epitopes and/or major parts of said epitopes induce an immune reaction (antibody synthesis) when they are injected into a rabbit.

[0040] Said sequences are unexpectedly characterized

by substantial immunogenicity towards monoclonal and polyclonal antibodies, but are sufficiently short to be readily and advantageously obtained by synthesis.

[0041] The present invention also relates to the conformational epitopes which comprise at least two different sequence epitopes and/or at least two major parts of said epitopes according to the invention and above identified.

[0042] The conformational epitopes are made up of two or more different portions of a polypeptide sequence, which portions are located in proximity to each other when the protein is folded in its tertiary or quaternary structure.

[0043] These epitopes are capable of being "recognized" (that is to say of exhibiting an immunoaffinity), preferably simultaneously, with inhibitors of factor VIII, in particular B and T lymphocytes (by way of the major histocompatibility locus (MHC I and/or II)) and/or anti-factor VIII antibodies (Scandella et al. (2000); Reding et al. (2000)).

[0044] Preferably, the said epitopes and/or the major parts of said epitopes are complexed with a carrier protein or a carrier peptide, such as BSA, or KLH haemocyanin, as to form a complex exhibiting a more powerful immunogenicity.

[0045] The present invention is also related to a pool of antigenic epitopes of factor VIII which comprises a mixture of the epitopes above mentioned linear epitopes (SEQ ID 10, SEQ ID 17, SEQ ID 20, SEQ ID 21, SEQ ID 24, SEQ ID 27, SEQ ID 30, SEQ ID 31, SEQ ID 32, SEQ ID 33) or conformational epitopes made of said epitopes or a pool which may comprise at least one of said epitopes and one or more additional antigenic epitopes of factor VIII already described in the state of the art and preferably selecting

from the group consisting of :

- the epitope arginine 1648 to tyrosine 1664 inclusive, defined by the following sequence:

SEQ ID No:1:

Arg Asp Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp
1 5 10 15
Tyr

and possibly deleted from one or more amino acids of the tetrapeptide Arg-Asp-Ile-Thr (P7), or one or two of the last amino acids of the dipeptide Asp-Tyr

- the epitope aspartic acid 1681 to arginine 1696 (P8) inclusive, defined by the following sequence:

SEQ ID No:2:

Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
1 5 10 15

possibly deleted from one or more amino acids of the epitope Asp-Glu-Asp-Glu,

- the epitope threonine 1739 to tyrosine 1748 inclusive, defined by the following sequence:

SEQ ID No:3:

Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr
1 5 10

- the epitope asparagine 1777 to phenylalanine 1785 inclusive, defined by the following sequence:

SEQ ID No:4:

Asn Gln Ala Ser Arg Pro Tyr Ser Phe
1 5

possibly deleted from one or more amino acids of the terminal dipeptide Ser-Phe or tetrapeptide Pro-Tyr-Ser-Phe

- the epitope glutamic acid 1794 to tyrosine 1815 inclusive, defined by the following sequence:

SEQ ID No:5:

Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro
1 5 10 15
Asn Glu Thr Lys Thr Tyr

20

possibly deleted from one or more amino acids of the first tripeptide Glu-Asp-Gln (P9) or the first nonapeptide Glu-Asp-Gln-Arg-Gln-Gly-Ala-Glu-Pro

- the epitope methionine 1823 to aspartic acid 1831 inclusive, defined by the following sequence:

SEQ ID No:6:

Met Ala Pro Thr Lys Asp Glu Phe Asp
1 5

- the epitope glutamic acid 1885 to phenylalanine 1891 inclusive, defined by the following sequence:

SEQ ID No:7:

Glu Thr Lys Ser Trp Tyr Phe
1 5

- the epitope glutamic acid 1885 to alanine 1901 inclusive, defined by the following sequence:

SEQ ID No:8:

Glu Thr Lys Ser Trp Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala
1 5 10 15

possibly deleted from one or more amino acids from the heptapeptide Glu-Thr-Lys-Ser-Trp-Phe-Thr or from the tripeptide Cys-Arg-Ala.

- the epitope aspartic acid 1909 to arginine 1917 inclusive, defined by the following sequence:

SEQ ID No:9:

Asp Pro Thr Phe Lys Glu Asn Tyr Arg
1 5

- the epitope alanine 108 to valine 128 inclusive, defined by the following sequence:

SEQ ID No:11:

Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys
1 5 10 15
Glu Asp Asp Lys Val
20

possibly deleted from the terminal amino acids alanine and valine (P1)

- the epitope glutamic acid 181 to leucine 192 inclusive, defined by the following sequence:

SEQ ID No:12:

Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
1 5

possibly deleted from one or two amino acids of the terminal dipeptide Thr-Leu

- the epitope aspartic acid 203 to alanine 227 inclusive, defined by the following sequence:

SEQ ID No:13:

Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln
1 5 10 15
Asp Arg Asp Ala Ala Ser Ala Arg Ala
20 25

possibly deleted from one or more amino acids of the nonapeptide Asp-Arg-Asp-Ala-Ala-Ser-Ala-Arg-Ala

- the epitope aspartic acid 327 to methionine 355 inclusive, defined by the following sequence:

SEQ ID No:14:

Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu
1 5 10 15
Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met
20 25

possibly deleted from one or more amino acids from the terminal dipeptide Asp-Ser or the octapeptide Asp-Asp-Leu-Thr-Asp-Ser-Glu-Met (P2).

- the epitope aspartic acid 403 to lysine 425 inclusive, defined by the following sequence:

SEQ ID No:15:

Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg
1 5 10 15
Ile Gly Arg Lys Tyr Lys Lys
20

possibly deleted from one or more amino acids of the tetrapeptide Asp-Asp-Arg-Ser (P3),

- the epitope valine 517 to arginine 527 inclusive, defined by the following sequence:

SEQ ID No:16:

Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg
1 5 10

possibly deleted from one or the two amino acids of the dipeptide Pro-Arg,

- the epitope histidine 693 to glycine 701 inclusive, defined by the following sequence:

SEQ ID No:18:

His Asn Ser Asp Phe Arg Asn Arg Gly
1 5

- the epitope serine 710 to aspartic acid 725 inclusive, defined by the following sequence (P4):

SEQ ID No:19:

Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Gly Asp Ser Tyr Glu Asp
1 5 10 15

- the epitope isoleucine 2081 to serine 2095 inclusive, defined by the following sequence:

SEQ ID No:22:

Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser
1 5 10 15

possibly deleted from one or more amino acids from the tetrapeptide Ile-His-Gly-Ile

- the epitope tyrosine 2105 to glycine 2121 inclusive, defined by the following sequence:

SEQ ID No:23:

Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr
1 5 10 15
Gly

possibly deleted from one or more amino acids of the tripeptide Tyr-Ser-Leu (P10)

- the epitope glutamine 2235 to leucine 2251 inclusive, defined by the following sequence (P13):

SEQ ID No:28:

Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser
1 5 10 15
Leu

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu or one or more amino acids of the tetrapeptide Val-Lys-Ser-Leu

- the epitope glycine 2242 to leucine 2251 inclusive, defined by the following sequence:

SEQ ID No:29:

Gly Val Thr Thr Gln Gly Val Lys Ser Leu
1 5 10

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu, said epitope presenting a possible partial overlapping with a known monoclonal antibody binding site ESH8 2248-2285

[0046] Another aspect of the present invention relates to an inhibitor of factor VIII which exhibits an immunoaffinity with antigenic epitopes, with the major parts of said epitopes and/or with the complex according to the invention.

[0047] An inhibitor is understood to mean any biological molecule or cell (such as a T-lymphocyte) binding to said FVIII and capable of giving rise to immune disorders (characterized by humoral immune response and/or cellular immune response against said FVIII).

[0048] In particular, such an inhibitor can be an anti-factor VIII monoclonal or polyclonal antibody or antibody fragment (such as the hypervariable Fab portion of the said antibody) which inactivates the said factor VIII and/or which inhibits the binding of factor VIII to the von Willebrand factor and/or to membrane phospholipids.

[0049] Advantageously, the said inhibitors are synthesized by a "chimaeric" animal which comprises a human immune system, such as an hu-SCID mouse or transgenic mouse

producing human antibodies or other antibodies production technologies as phage display technology or immortalized B-cells, by EPV in particular.

[0050] Another aspect of the invention relates to an anti-inhibitor which is directed against the said previously described factor VIII inhibitor.

[0051] An anti-inhibitor which is directed against the factor VIII inhibitor is understood to mean any chemical or biological molecule, a cell and/or a cell fragment (receptor) which is capable of interfering with the said inhibitor in such a way as to ensure its inactivation or avoid or reduce its binding to the factor VIII.

[0052] Preferably, such an anti-inhibitor is an anti-anti-factor VIII idiotype (monoclonal or polyclonal) antibody or antibody fragment, natural or obtained by genetic engineering.

[0053] Another aspect of the invention relates to a pharmaceutical composition which comprises an adequate pharmaceutical carrier or a diluant and an element selected from the group consisting of said epitopes or a pool thereof, an inhibitor of factor VIII which is directed against them, an anti-inhibitor which is directed against the said inhibitor, and/or a mixture of these.

[0054] The type and amount of adequate pharmaceutical carrier or diluant (and possibly adjuvant or excipient) present in said pharmaceutical composition, may vary according to the method of administration and is possibly combined an adjuvant in order to improve therapeutical properties of the pharmaceutical composition according to the invention or to reduce its possible side effects. Suitable pharmaceutical acceptable carriers used in the pharmaceutical composition according to the invention are well known by the person skilled in the art

and are selected according to the methods generally applied by pharmacists and may include solid, liquid or gaseous non-toxic pharmaceutically acceptable carriers. The percentage of active product / pharmaceutical acceptable carrier may vary within very large ranges only limited by the tolerance and the possible side effects on patients (including humans), and by frequency and/or mode of administration.

[0055] Another aspect of the invention relates to a diagnostic and/or purification device, such as a diagnostic kit, an affinity filter, or a chromatography column which comprises an element which is selected from the group consisting of these epitopes and/or major parts of said epitopes, the complex according to the invention or a pool thereof, an inhibitor which is directed against them, an anti-inhibitor which is directed against said inhibitor, and/or a mixture of these. Advantageously, said device comprises the pool of said epitopes which allow a screening of patients and may detect the most important inhibitors present in said patients and which allow a positive test with enough specificity and sensibility.

[0056] The purification device can therefore consist of a chromatography column which comprises these epitopes and/or major parts of epitopes, attached to the solid phase of the chromatography column.

[0057] A physiological liquid (such as serum), which is derived from a patient and which comprises inhibitors of factor VIII pass through a solid support (chromatography column), with said inhibitors (for example antibodies) becoming attached specifically to said epitopes or said major parts or a pool thereof. Following elution, it is possible to collect said inhibitors by causing them to react with anti-inhibitors (anti-anti-factor VIII idiotype antibodies).

[0058] It is also possible to characterize the anti-anti-factor VIII idiotype antibodies which are present in a serum by these anti-inhibitors passed through a solid support (chromatography column) on which inhibitors of factor VIII have been attached to the solid phase.

[0059] It is also possible to reinject (*ex vivo* treatment) the physiological liquid (blood or serum or a derived fraction) to said patient after its inhibitors of factor VIII have been removed by binding with said epitopes or a pool thereof; said inhibitors being removed from the physiological fluid (blood or serum) similarly as proposed for dialysis method applied to human patients.

[0060] The present invention is also related to a method of treatment (*ex vivo* treatment) of a patient suffering from a pathology induced by inhibitors to the factor VIII which comprises the steps of extracting said physiological liquid (blood or serum) from the patient, obtaining its reaction upon a solid support binding the epitopes or a pool thereof according to the invention and reinjecting said physiological liquid to the patient after the removing of the inhibitors having fixed said epitopes, majors parts or a pool thereof.

[0061] A final aspect of the invention relates to the use of the pharmaceutical composition according to the invention for preparing a medicament used for preventing and/or treating immune disorders, in particular those induced by inhibitors of factor VIII, inhibitors of the binding of factor VIII to the factor IX and/or the factor X and/or the von Willebrand factor (vWF) and/or inhibitors of the binding of factor VIII to membrane phospholipids.

[0062] The present invention will be described in details in the following non-limiting examples in reference to the enclosed figures.

Brief description of the figures

[0063] Figure 1 depicts the hydrophilicity, flexibility and accessibility graph of the A3 sequence of Factor VIII renumbered 1 to 371 amino acids (surface value for each amino acid).

[0064] Figure 2a represents the elution profile related to the purification of human anti-SEQ ID 32 antibodies by affinity chromatography on peptide-Sepharose column. Cohn fraction II+III solution (50 ml) was loaded onto the column (1 ml gel) at a flow rate of 1 ml/min. The separation of specific antibodies was performed as described hereafter. The arrow indicates the position of specific human anti-SEQ ID 32 antibodies.

[0065] Figure 2b represents FVIII clotting activity in the presence of anti-(SEQ ID 32) IgG purified from Cohn fraction II+III. The clotting activity of FVIII was measured as described hereafter in the presence of increasing amount of anti-SEQ ID 32. The % of FVIII activity =(FVIII activity in the presence of antibody/FVIII activity in absence of antibody)*100.

[0066] Figure 3 represents the human anti-peptide antibody immunoreactions with FVIII polypeptides after western blotting (panel A from left to right : human antibodies HAP1 through HAP4, specific for different FVIII epitope sequences found in the FVIII HC - see also table 2 and panel B : human antibodies specific for the P5 peptide and the FVIII LC sequences, P7, P8 and P9 - see also table 2). The RAP9 lane shows the reactivity of FVIII polypeptides towards purified rabbit antibodies specific for the peptide sequence Arg^{1797}-Tyr^{1815} (see also table 2).

[0067] Figure 4 represents ELISA reactivity of 4 inhibitor plasmas with different peptide sequences. Inhibitors present in 4 patients plasmas were analyzed by

ELISA test using as coated antigens the different selected FVIII epitopes synthetic peptides as indicated in ordinate.

Examples

Materials and Methods

Reagents

[0068] MAS530p (Harlan-Seralab, Indianapolis, IN) is a mouse monoclonal antibody specific for the 44-kDa A2 domain of the factor VIII heavy chain. Biotin-labeled rabbit IgG anti-mouse IgG was purchased from Dakopatts (Copenhagen, Denmark). Biotin-labeled goat IgG anti-human IgG and biotin-labeled mouse IgG anti-rabbit IgG were obtained from Sigma Chemicals (St Louis, MI), purified α-thrombin (3000 IU/mg), streptavidin-peroxidase conjugate, ovalbumin (OVA), bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), and o-phenylenediamine (OPD) were purchased from Sigma Chemicals (St. Louis, MI). Casein was obtained from Merck (Darmstadt, Germany). 4-chloro-1-naphtol and biotinylated molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA). Freund's adjuvant was from Difco (Detroit, Michigan).

FVIII concentrates

[0069] Plasma FVIII (p-FVIII) was a solvent/detergent-treated FVIII concentrate (100 IU/mg protein) purified by ion exchange chromatography (FVIII Conc. SD, CAF-DCF- Red Cross , Brussels, Belgium). Albumin-free recombinant FVIII (rFVIII) was obtained from Hyland (Glendale, CA).

Plasma fraction immunoglobulins

[0070] Cohn Fraction II+III was obtained from large plasma pool from 4,800 unpaid donors, after precipitation in the presence of increasing ethanol concentration. This fraction contains all Ig classes and subclasses. IgG composition was determined by nephelometry. The relative percentage of each subclass was 63,7; 30,1; 3,4 and 2,8 for IgG1, IgG2, IgG3 and IgG4 respectively (average values for 3 different batches of FII+III).

Factor VIII concentrates, Factor VIII activity and activity inhibition

[0071] Factor VIII activity was determined in a one-stage clotting assay adapted for use on the Coagulometer KC4A (Sigma Diagnostics). The assay uses severe hemophilia A plasma (Organon Teknika, Cambridge, UK) and APPT reagent from Instrumentation Laboratory (Warrington, UK). Potencies were calculated relative to the 5th International Standard FVIII concentrate 88/640 (5.4 IU/ml) (NIBSC, Potters Bar, UK). FVIII-inhibitory activity was measured in purified rabbit and human IgG preparations according to the modified Bethesda assay. Briefly, affinity-purified IgGs were serially diluted and incubated for 1 h in the presence of FVIII concentrate 88/640 (1 IU/ml) at 37°C. The residual FVIII activity was measured as described above.

[0072] Activation of factor VIII by α-thrombin and immunoblotting has been described elsewhere (Peerlinck et al, 1997).

[0073] Synthesis of peptides, conjugation of peptides to carrier proteins and production of rabbit anti-peptide antisera were performed by Neosystem (Strasbourg, France).

Purification of rabbit and human antibodies by affinity chromatography

[0074] For purification of rabbit and human antibodies, 5 mg of each different peptide was coupled to 1 ml pre-packed NHS-activated Sepharose (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Specific anti-peptide antibodies were purified with an automated liquid chromatography system (ÄKTAexplorer 100A, Pharmacia Uppsala, Sweden) either from 50 ml rabbit antiserum or from 100 ml of a human plasma fraction, obtained after Cohn fractionation (fraction II+III; 13 mg protein/ml). Briefly, samples were dialyzed 3 times against 5 volumes of TE buffer (20 mM Tris-HCl pH 7.2, 150 mM NaCl and 0.02% NaN_3) and loaded onto the column at a flow rate of 1 ml/min. The column was sequentially washed at 2 ml/min with 50 ml TE buffer and 30 ml TE containing 1 M NaCl. After absorption, the material was eluted (1 ml/min) with 5 ml of 0.1 M citric acid pH 2.5 and directly recovered in 5 ml of 1M Tris-HCl, pH 9.0. Samples were finally dialyzed versus 10 volumes of equilibration buffer and concentrated on Centriprep-30 (Amicon, Beverly, MA). Ig recovery was determined by the Bio-Rad protein assay.

Results

Selection of potential factor-VIII linear epitopes

[0075] More than 30 surface regions (linear epitopes) spanning 8 to 25 residues, characterized by a high hydrophilicity, flexibility and accessibility were identified on the FVIII molecule. On the basis of their high probability of an outer location (see Fig. 1 for A3), 16 linear peptides (P1 to P16) were selected, matching identified stretches of 13 or more amino-acid residues. These peptides were synthesized and coupled to ovalbumin

for production of specific antiserum (Table 1, hereafter). P8 includes the epitope described by Shima et al (1988) and was used as an external control.

Experimental results obtained from said synthesized linear epitopes using the rabbit model

[0076] Results are summarized in Table 1 which concerns the characterization of rabbit anti-FVIII-peptide antisera and recovered affinity-purified of immunoglobulins.

[0077] Sixteen synthetic peptides (from 10 to 20 amino acids) were selected in the A, B, C1 and C2 domains. After conjugation with ovalbumin, the OVA-peptide conjugates were injected into rabbits and FVIII anti-peptide antisera RAP1 to RAP16 were studied.

[0078] More precisely, two rabbits were immunized with each FVIII-peptide-ovalbumin preparation. Specific antisera RAP1 to RAP16 (column b, Table 1) were prepared and assayed in an ELISA (column c, Table 1) using rFVIII or FVIII-peptide-KLH as the antigen. ELISA titre is expressed as the negative log of the reciprocal of the serum dilution giving 50% binding. The immunoglobulins were then purified by chromatography on peptide-bound Sepharose. The FVIII domain recognized by the anti-FVIII peptide Ig after immunoblotting is shown in (column d, Table 1) and Ig protein recoveries (column e, Table 1) were measured using immunoglobulins as the standard. The inhibitory activity, expressed in BU/mg protein, was determined in a FVIII neutralizing activity assay (column f, Table 1).

Immunogenicity of FVIII peptides and characterization of rabbit anti-FVIII peptide antisera

[0079] The reactivity of FVIII anti-peptide antisera was measured by an ELISA using, as antigen, either the

different corresponding FVIII-peptide coupled to KLH protein or purified rFVIII. The binding reaction of each anti-FVIII-peptide antiserum was specific both for the FVIII peptide used to elicit the immune response in rabbit and for rFVIII (see Table 1).

[0080] To demonstrate the FVIII epitope specificity of the rabbit anti-peptide antibodies, rFVIII and the rFVIII fragments obtained after treatment with thrombin were resolved by SDS-PAGE and analyzed by western blotting with the different preparations of rabbit IgGs. As expected, most antisera (14/16, 87%), showed a strong reaction with the corresponding FVIII fragment containing the selected linear epitope (see Table 1).

Purification of rabbit-anti-FVIII peptide antibodies

[0081] The specific rabbit IgG were purified by affinity chromatography on peptide-Sepharose as described under Methods. When FVIII-neutralizing activity was measured in a one-stage clotting assay, significant inhibition was found with two rabbit IgG purified preparations: RAP2, corresponding to IgG specific for SEQ ID No. 14 and RAP7 specific for SEQ ID No: 01.

Epitope mapping of rabbit anti-FVIII peptide antibodies by immunoblotting with human rFVIII

[0082] To demonstrate the FVIII epitope specificity of the rabbit anti-peptide antibodies, rFVIII and the rFVIII fragments obtained after treatment with thrombin were resolved by SDS-PAGE and analyzed by western blotting with different preparations of rabbit IgGs (RAP1 to RAP17 Igs).

[0083] In each run, the rFVIII heavy chain (HC) and light chain (LC) and their thrombin proteolysis products (44 kDa and 72 kDa) were identified with a mixture of two

monoclonal antibodies, MoAb 530p and MoAb18, respectively specific for the heavy and light chain. MoAb18 recognizes the NH_2-terminal light-chain FVIII fragment obtained after thrombin activation, which proved too small to remain in the gel after electrophoresis. Fourteen of the 17 rabbit immunoglobulin preparations reacted strongly with both rFVIII and pFVIII. Antisera RAP1, RAP2, RAP3, RAP4 recognized exclusively the heavy chains (200 kDa to 92 kDa). Antisera RAP1 and RAP2 reacted with the 50-kDa A1-domain fragment; RAP3 and RAP4 bound to the 44-kDa fragment (domain A2); RAP5 (specific for the B domain) bound to the high-molecular-weight FVIII heavy chain (about 200-kDa).

[0084] RAP7, RAP8, and RAP9 reacted with the 80-kDa light-chain doublet. RAP9 and RAP12 to RAP17 antibodies also detected the 72-kDa FVIII light-chain fragment. As expected, each reactive antiserum showed a strong reaction with the corresponding FVIII fragment containing the selected linear epitope. No reaction was detectable in the gels between RAP6 or RAP10 and the HC or LC FVIII fragments.

Experimental results obtained from said synthesized linear epitopes to purify and characterize human autoantibodies

[0085] Table 2 concerns the characterization of human anti-FVIII antibodies from Cohn fraction II+III of healthy individuals.

[0086] Human anti-peptide IgG preparations (HAP1 through HAP17) were so far purified on Sepharose coupled to 13 different FVIII peptides (column a, Table 2). The Igs (column b, Table 2) were analyzed by immunoblotting. Binding to the rFVIII HC or LC chains and to the rFVIII thrombin fragment is shown respectively in columns c and d, Table 2. FVIII-domain reactivity is shown in column e,

Table 2. Arrows indicate a decrease in 80-kDa band intensity. Ig recovery (column f, Table 2) after affinity purification is expressed in μg/10 mg loaded FII+III (see Materials and Methods). Inhibition of the clotting assay was determined after incubation in the presence of each of the 13 Ig preparations in the Bethesda assay (column g, Table 2).

Use of FVIII peptides for the immunopurification of human anti-FVIII antibodies in healthy donors

[0087] To prepare and characterize human anti-FVIII antibodies present in healthy individuals, we analyzed Cohn fraction II+III, rich in immunoglobulins, for the presence of selected specific anti-peptide antibodies. Human anti-FVIII-peptide antibodies (HAP1 to HAP11, HAP16 and HAP17) were purified by affinity chromatography on Sepharose coupled to the appropriate peptide (see Table 2). As a typical example, figure 2 shows the chromatographic profile obtained with SEQ ID 32, a sequence found in C2 domain. Table 2 summarizes the results obtained with 17 epitopic sequences selected in each FVIII domain (A1, A2, A3, B, C1 and C2). Significant amounts of immunoglobulins, specific for each of the 13 FVIII peptides used, were obtained from the starting plasma fraction II+III. The specificity of the resulting purified human antibodies was directly tested by immunoblotting with plasma FVIII, recombinant FVIII, and the fragments obtained after thrombin proteolysis (see Table 2).

[0088] The IgG isotype distribution in the human purified antibody preparations was found to be quite heterogeneous. Interestingly, 40 to 79% of the recovered IgGs belonged to the IgG2 subclass. In most preparations, IgG4 appeared to be over-represented (up to 25%).

[0089] All the human anti-FVIII-peptide antibody preparations were tested for the capacity to inhibit FVIII activity in a one-stage clotting assay. Table 2 shows that seven out of 13 preparations tested (54%) displayed inhibitory activity, SEQ ID No: 14, SEQ ID No: 19, SEQ ID No: 2, SEQ ID No: 5, SEQ ID No: 22, SEQ ID No: 32 and SEQ ID No: 33, respectively. As a typical example, the inhibition of FVIII activity in function of anti-SEQ ID 32 Ig concentration is shown in figure 2.

Human anti-FVIII-peptide Ig immunospecificity towards FVIII

[0090] The specificity of the resulting purified human antibodies was tested by immunoblotting with plasma FVIII, recombinant FVIII, and the fragments obtained after thrombin proteolysis. Again, the FVIII fragments were identified with either FVIII-HC- or FVIII-LC-specific mouse monoclonal antibodies or FVIII-peptide-specific rabbit polyclonal antibodies. The human antibodies were identified after binding of biotinylated goat anti-human IgG. Figure 3 shows the immunoreaction of high-molecular-weight FVIII (≥ 92-kDa) with four human antibody preparations, purified on Sepharose coupled to FVIII peptide SEQ ID No: 11 (Ser^{109}-Lys^{127}), SEQ ID No: 14 (Cys^{329}- Asp^{348}), SEQ ID No: 15 (Tyr^{407}-Lys^{425}) or SEQ ID No: 19 (Cys^{711}-Asp^{725}). The 50-kDa FVIII fragment (domain A1) was recognized by human antibodies purified on Ser^{109}- Lys^{127} or Cys^{329}-Asp^{348}-Sepharose and the 44-kDa FVIII fragment (A2) by immunoglobulins purified on Tyr^{407}-Lys^{425} and Cys^{711}-Asp^{725}-Sepharose. The lack of reactivity of the anti-(Ser^{817}-Ser^{830}) immunoglobulin preparation (HAP5) with the FVIII fragments confirms that this epitope is located in the amino-terminal end of domain B (Figure 3). Human antibodies purified on Sepharose coupled to peptide SEQ ID No: 1 (Arg^{1652}-Tyr^{1664}) or

SEQ ID No: 2 (Asp^{1681}-Arg^{1696}) reacted strongly with the 80-kDa FVIII light chain (Figure 3). For both preparations, the reaction with the 80-kDa band disappeared after thrombin proteolysis, indicating that the epitopes, as expected, are located in the *a3* acidic peptide at the NH_2-terminal part of the FVIII A3 domain. When human antibodies specific for peptide SEQ ID No: 5 (Arg^{1797}-Tyr^{1815} in A3 domain) were analyzed by immunoblotting, their specificity for rFVIII appeared restricted to the 80-kDa FVIII light chain and its 72-kDa thrombin fragment.

[0091] No immunoreaction with the rFVIII chains or fragments was detected with antibody preparations specific for FVIII peptides SEQ C and SEQ ID No: 23, although a positive reaction was obtained in the ELISA using rFVIII. This could mean that these immunoglobulin preparations recognize a conformational epitope.

Use of FVIII synthetic peptides to characterize human anti-FVIII antibodies in hemophilia A patient plasmas

[0092] The selected peptides were used in ELISA experiment to determine the anti-FVIII antibody specificity's present in hemophilia A plasmas. The peptides were coated on microplate (25 µg/ml in PBS buffer during 16h at 4°C). A 1/10 to 1/1000 dilution of plasma patient in Tris-casein buffer was reacted with the coated peptide for 2h at 37°C. The bound human IgG was measured as described in Methods. Control samples were plasma pools of healthy donors. Figure 4 shows the results obtained with the plasma of 4 hemophilia A patients. The optical densities are corrected average values (OD patient-OD normal plasma pool) of two independent experiments.

Molecular model epitope prediction

[0093] Pemberton et al (1997) have built a molecular model of the A domains of FVIII. This 3-D model makes it possible to explore predictions for important regions of FVIII activity. The model was used to locate the FVIII-peptide epitopes identified by the Parker and Hodge algorithms. As predicted by these algorithms, all peptides located in the A domains were found on the FVIII surface and were fully accessible to specific.

[0094] The overlap between the epitope and the FIXa-binding loop (5 common residues spanning Glu^{1811}-Tyr^{1815}) may explain the inhibitory action of the corresponding anti-(Arg^{1797}-Tyr^{1815}) antibodies on formation of the fibrin clot.

Analysis of the results

[0095] In the clotting test, significant inhibition of FVIII activity was recorded in the presence of rabbit anti-(Cys^{329}-Asp^{348}) and anti-(Arg^{1653}-Tyr^{1664}) antibodies, but different inhibition patterns were observed. Inhibition by anti-(Arg^{1653}-Tyr^{1664}) follows second-order kinetics with a drastic reduction in FVIII activity. Anti-(Cys^{329}-Asp^{348}) Ig is less efficient and shows a more complex type of reaction, with a non-linear dependence on the antibody concentration. Epitope Arg^{1652}-Tyr^{1664} and the adjacent major binding site vWF (residues Glu^{1675}-Glu^{1684}) are located in the acidic light-chain peptide *a3*. As shown by western blotting, *a3* is released from the A3 domain after thrombin treatment, preventing further binding of anti-(Arg^{1652}-Tyr^{1664}) Ig to activated FVIII. Similar results have been reported by Shima et al (1991), who described the FVIII sequence Asp^{1663}-Ser^{1669} as a binding site of rabbit polyclonal antibodies neutralizing FVIII activity. Epitope Cys^{329}-Asp^{348} overlapped the acidic Asp^{348}-Lys^{362} sequence (in *a1*) described as adjacent to the activated protein C

(Arg^{336}) and thrombin (Arg^{372}) cleavage sites. It is the target of human hemophilic inhibitors. Anti-(Asp^{348}-Lys^{362}) antibodies may interfere with proteolysis or with the FX interaction site (Met^{337}-Arg^{372}) (Saenko et al., 1999 and Scandella et al., 2000).

[0096] FVIII-neutralizing activity was measured in all 13 Ig preparations. Seven human Ig preparations displayed inhibition of procoagulant activity, these being specific for amino-acid residues Cys^{711}-Asp^{725}, Tyr^{1681}-Arg^{1696}, and Arg^{1797}-Tyr^{1815} respectively. The Cys^{711}-Asp^{725} sequence contains sulfated tyrosines at Tyr^{718}, Tyr^{719}, and Tyr^{723}, and overlaps with the FVIII HC region Lys^{713}-Arg^{740} described as promoting both activation and HC proteolysis. The additional sulfated groups may be required for proper interaction with thrombin or another component as in the FX-activating complex. The sequence also overlaps with region Gly^{701}-Ser^{750}, recognized by a weakly inhibitory mouse monoclonal antibody. Peptide P8 (Tyr^{1681}-Arg^{1696}) (FVIII LC) includes the sequence Glu^{1684}-Arg^{1689} already described by Shima et al, 1991. It contains the thrombin activation site Arg^{1689}-Ser^{1690}. P4 (Cys^{711}-Asp^{725}) is also included in the Asp^{712}-Ala^{736} sequence detected by analysis of the patient antibody repertoire by gene phage display technology. It is proposed as a possible additional inhibitor in patients (van den Brink et al, 2000). Peptide P9 (Arg^{1797}-Tyr^{1815}) contains the FXa binding site (see below).

[0097] Of the 16 anti-FVIII-peptide immunoglobulins purified from humans or produced in rabbits, 7 did neutralize FVIII activity under the tested conditions. Using small peptide sequences and immunobinding assays, we have provided evidence for additional new epitopes. We have located new epitopes in the A1 domain (residues Ser^{109}-Cys^{127}), the A2 domain (Cys^{407}-Lys^{425}), and the B domain (Ser^{817}-Ser^{830} and Glu^{1078}-Pro^{1092}).

[0098] Autoantibodies immunopurified with denatured FVIII have been reported in healthy subjects and in pools of normal human immunoglobulins (processed fraction II, see above) (Algiman et al., 1992 and Moreau et al., 2000). A possible role in clearance of denatured FVIII or its fragments from the bloodstream and/or in the immunotolerance was suggested.

[0099] Identification of the FVIII epitopes is a major challenge to be met in order to improve FVIII treatment and the quality of therapeutic FVIII concentrates. FVIII epitope sequences help to determine the contribution of patient polyclonal anti-FVIII Igs to overall inhibitory and regulatory activity. They could also be used to monitor the usual switch in anti-FVIII specificity in a patient during treatment. Said characterization of FVIII epitopes and a model of their locations on the folded molecule improves the treatment of inhibitors in both hemophilic and non- hemophilic patients (detection, follow-up, therapeutic use of FVIII epitope peptides...).

Table 1 Characterization of rabbit anti-FVIII peptides antibodies

SEQ ID(a)	Rabbit Antiserum(b)	ELISA Titre(c)		FVIII domain recognize(d)	RAP-IgG Recovery(e) µg/ml serum	Inhibitor Titre(f) BU/mg
		P-KLH	r-FVIII			
SEQ ID 11	RAP1	2.5	2.2	A1	27	-
SEQ ID 14	RAP2	3.6	2.5	A1/a1	55	1,5
SEQ ID 15	RAP3	2.5	3.2	A2	268	-
SEQ ID 19	RAP4	2.5	1.3	A2/a2	12	-
SEQ ID 21	RAP5	4.6	3.9	B	106	-
SEQ C	RAP6	3.8	2.9	-	14	-
SEQ ID 01	RAP7	3.9	3.9	a3 ↓	35	0,5
SEQ ID 02	RAP8	1.9	0.9	a3/A3 ↓	3	-
SEQ ID 05	RAP9	3.8	2.6	A3	42	-
SEQ ID 23	RAP10	3.9	0.8	-	65	-
SEQ ID 22	RAP11	ND	ND	ND	ND	ND
SEQ ID 26	RAP12	4,1	1,1	C2	ND	ND
SEQ ID 27	RAP13	3,7	1,1	C2	ND	ND
SEQ ID 28	RAP14	3,8	0,9	C2	ND	ND
SEQ ID 31	RAP15	3,2	0,7	C2	ND	ND
SEQ ID 32	RAP16	3,5	1,8	C2	ND	ND
SEQ ID 33	RAP17	4,8	1,2	C2	ND	ND

Table 2 Characterization of human anti-FVIII peptides autoantibodies

SEQ ID(a)	Human Anti-peptide Ig(b)	FVIII reactivity on immunoblot (-thrombin)(c)	(+thrombin)(d)	FVIII domain(e)	HAP-IgG Recovery(f) µg/10 mgIgG	FVIII inhibitory Activity(g) BU/mg
SEQ ID 11	HAP1	>92kDa	50kDa	A1	0,27	-
SEQ ID 14	HAP2	>92kDa	50kDa	A1/a1	1,07	3,4
SEQ ID 15	HAP3	>92kDa	44kDa	A2	0,06	-
SEQ ID 19	HAP4	92kDa	44kDa	A2/a2	0,12	+
SEQ ID 21	HAP5	>100kDa	-	B	0,26	-
SEQ C	HAP6	-	-	-	0,03	-
SEQ ID 01	HAP7	80kDa	80kDa	a3 ↓	0,20	-
SEQ ID 02	HAP8	80kDa	80kDa	a3/A3 ↓	0,01	+
SEQ ID 05	HAP9	80kDa	72kDa	A3	0,08	+
SEQ ID 23	HAP10	-	-	-	0,11	-
SEQ ID 22	HAP11	ND	ND	ND	0,98	4,3
SEQ ID 26	HAP12	ND	ND	ND	ND	ND
SEQ ID 27	HAP13	ND	ND	ND	ND	ND
SEQ ID 28	HAP14	ND	ND	ND	ND	ND
SEQ ID 31	HAP15	ND	ND	ND	ND	ND
SEQ ID 32	HAP16	80kDa	72 kDa	A3C1C2	2,40	6,3
SEQ ID 33	HAP17	ND	ND	ND	1,06	2,4

+: Inhibition >25% at 100µg/ml

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CLAIMS

1. An antigenic epitope of FVIII polypeptide sequence, which is selected from the group consisting of :

- the epitope comprised between serine 2018 and histidine 2031 inclusive, defined by the following sequence:
  SEQ ID No:10:
  Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His
  1 5 10
- the epitope tyrosine 555 to glutamine 565 inclusive defined by the following sequence:
  SEQ ID No:17:
  Tyr Lys Glu Ser Val Asp Gly Arg Gly Asn Gln
  1 5 10
- the epitope leucine 730 to serine 741 inclusive, defined by the following sequence:
  SEQ ID No:20:
  Leu Leu Ser Lys Asn Asn Ala Ile Glu Pro Arg Ser
  1 5 10
  possibly deleted from the terminal amino acid serine and/or the first amino acid leucine
- the epitope serine 817 to serine 830 inclusive, defined by the following sequence:
  SEQ ID No:21:
  Ser Asp Asp Pro Ser Gly Ala Ile Asp Ser Asn Asn Ser
  1 5 10
- the epitope asparagine acid 2128 to asparagine acid 2138 inclusive, defined by the following sequence:
  SEQ ID No:24:
  Asn Val Asp Ser Ser Gly Ile Lys His Asn
  1 5 10
- the epitope serine 2204 to glutamine 2222 inclusive, defined by the following sequence:

SEQ ID No:27:

Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp
1 5 10 15
Arg Pro Gln

- the epitope isoleucine 2262 to glutamine 2270 inclusive, defined by the following sequence:

SEQ ID No:30:

Ile Ser Ser Ser Gln Asp Gly His Gln
1 5

- the epitope leucine 2273 to serine 2289 inclusive, defined by the following sequence:

SEQ ID No:31:

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
1 5 10 15
Ser

- the epitope proline 2292 to tyrosine 2305 inclusive, defined by the following sequence:

SEQ ID No:32:

Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr
1 5 10

possibly deleted from one or more amino acids of the terminal tripeptide Thr-Arg-Tyr

- the epitope glutamic acid 2322 to tyrosine 2332 inclusive, defined by the following sequence:

SEQ ID No:33:

Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
1 5 10

2.A pool of antigenic epitopes of factor VIII polypeptide sequence which comprise one or more antigenic sequence epitope of factor VIII according to claim 1 and possibly an antigenic sequence epitope of factor VIII which is selected from the group consisting of :

- the epitope arginine 1648 to tyrosine 1664 inclusive, defined by the following sequence:

SEQ ID No:1:

Arg Asp Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp
1 5 10 15
Tyr

possibly deleted from one or more amino acids of the tetrapeptide Arg-Asp-Ile-Thr or one or two of the last amino acids of the peptide Asp-Tyr.

- the epitope aspartic acid 1681 to arginine 1696 inclusive, defined by the following sequence:

SEQ ID No:2:

Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
1 5 10 15

possibly deleted from one or more amino acids of the epitope Asp-Glu-Asp-Glu.

- the epitope threonine 1739 to tyrosine 1748 inclusive, defined by the following sequence:

SEQ ID No:3:

Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr
1 5 10

- the epitope asparagine 1777 to phenylalanine 1785 inclusive, defined by the following sequence:

SEQ ID No:4:

Asn Gln Ala Ser Arg Pro Tyr Ser Phe
1 5

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Phe or the tetrapeptide Pro-Tyr-Ser-Phe.

- the epitope glutamic acid 1794 to tyrosine 1815 inclusive, defined by the following sequence:

SEQ ID No:5:

Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys Pro
1 5 10 15
Asn Glu Thr Lys Thr Tyr
20

possibly deleted from one or more amino acids from the first tripeptide Glu-Asp-Gln or the first nonapeptide Glu-Asp-Gln-Arg-Gln-Gly-Ala-Glu-Pro.

- the epitope methionine 1823 to aspartic acid 1831, defined by the following sequence:

SEQ ID No:6:

Met Ala Pro Thr Lys Asp Glu Phe Asp
1 5

- the epitope glutamic acid 1885 to phenylalanine 1891 inclusive, defined by the following sequence:

SEQ ID No:7:

Glu Thr Lys Ser Trp Tyr Phe
1 5

- the epitope glutamic acid 1885 to alanine 1901 inclusive, defined by the following sequence:

SEQ ID No:8:

Glu Thr Lys Ser Trp Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala
1 5 10 15

possibly deleted from one or more amino acids from the heptapeptide Gly-Thr-Lys-Ser-Trp-Phe-Thr or from the tripeptide Cys-Arg-Ala.

- the epitope aspartic acid 1909 to arginine 1917 inclusive, defined by the following sequence:

SEQ ID No:9:

Asp Pro Thr Phe Lys Glu Asn Tyr Arg
1 5

- the epitope alanine 108 to valine 128 inclusive, defined by the following sequence:

SEQ ID No:11:

Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys
1 5 10 15
Glu Asp Asp Lys Val
20

possibly deleted from the terminal amino acids alanine and/or valine

- the epitope glutamic acid 181 to leucine 192 inclusive, defined by the following sequence:

SEQ ID No:12:

Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
1 5

possibly deleted from one or two amino acids of the terminal dipeptide Thr-Leu

- the epitope aspartic acid 203 to alanine 227 inclusive, defined by the following sequence:

SEQ ID No:13:

Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln
1 5 10 15
Asp Arg Asp Ala Ala Ser Ala Arg Ala
20

possibly deleted from one or more amino acids of the nonapeptide Asp-Arg-Asp-Ala-Ala-Ser-Ala-Arg-Ala

- the epitope aspartic acid 327 to methionine 355 inclusive, defined by the following sequence:

SEQ ID No:14:

Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu
1 5 10 15
Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp Ser Glu Met
20 25

possibly deleted from one or more amino acids of the dipeptide Asp-Ser or the octapeptide Asp-Asp-Leu-Thr-Asp-Ser-Glu-Met.

- the epitope aspartic acid 403 to lysine 425 inclusive, defined by the following sequence:

SEQ ID No:15:

Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro Gln Arg
1 5 10 15
Ile Gly Arg Lys Tyr Lys Lys
20

possibly deleted from one or more amino acids of the tetrapeptide Asp-Asp-Arg-Ser.

- the epitope valine 517 to arginine 527 inclusive, defined by the following sequence:

SEQ ID No:16:

Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg
1 5 10

possibly deleted from one or the two amino acids of the dipeptide Pro-Arg

- the epitope histidine 693 to glycine 701 inclusive, defined by the following sequence:

SEQ ID No:18

His Asn Ser Asp Phe Arg Asn Arg Gly
1 5

- the epitope serine 710 to aspartic acid 725 inclusive, defined by the following sequence:

SEQ ID No:19

Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Gly Asp Ser Tyr Glu Asp
1 5 10 15

- the epitope isoleucine 2081 to serine 2095 inclusive, defined by the following sequence

SEQ ID No:22:

Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser
1 5 10 15

possibly deleted from one or more amino acids of the tetrapeptide Ile-His-Gly-Ile

- the epitope tyrosine 2105 to glycine 2121 inclusive, defined by the following sequence:

SEQ ID No:23:

Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr
1 5 10 15

Gly

possibly deleted from one or more amino acids of the tripeptide Tyr-Ser-Leu

- the epitope histidine 2152 to arginine 2163 inclusive, defined by the following sequence:

SEQ ID No:25:

His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg
1 5 10

- the epitope serine 2181 to asparagine 2198 inclusive, defined by the following sequence:

SEQ ID No:26:

Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr
1 5 10 15
Asn

possibly deleted from one or more amino acids from the terminal tripeptide Phe-Thr-Asn (P11)

- the epitope glutamine 2235 to leucine 2251 inclusive, defined by the following sequence:

SEQ ID No:28:

Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser
1 5 10 15
Leu

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu or one or more amino acids of the tetrapeptide Val-Lys-Ser-Leu.

- the epitope glycine 2242 to leucine 2251 inclusive, defined by the following sequence:

SEQ ID No:29:

Gly Val Thr Thr Gln Gly Val Lys Ser Leu
1 5 10

possibly deleted from one or two amino acids of the terminal dipeptide Ser-Leu

3. A conformational epitope which contains:

- either at least two different epitopes according to the claim 1 or,
- at least one epitope sequence according to claim 1 and an epitope selecting from the pool of epitopes sequences of claim 2.

4. A recombinant factor VIII having an amino acid sequence deleted from one of the antigenic epitopes sequences according to claim 1 or one or more epitopes sequences selected from the pool of epitopes of claim 2.

5. A complex comprising a carrier protein or a carrier peptide linked to an epitope sequence according to the claim 1 or 3.

6. An inhibitor of factor VIII polypeptide sequence which exhibits an immunoaffinity with the epitope sequence, the pool of epitopes sequences and/or the complex according to any one of the preceding claims 1 to 5.

7. The inhibitor according to Claim 6, which is an anti-factor VIII antibody or antibody fragment.

8. An anti-inhibitor, which is directed against the inhibitor of factor VIII according to Claim 6 or 7.

9. The anti-inhibitor according to Claim 8, which is an anti-anti-factor VIII idiotype antibody or antibody fragment.

10. A pharmaceutical composition, which comprises an adequate pharmaceutical carrier and at least one element selected from the group consisting of the epitope sequence, the pool of epitopes sequences, the complex, the recombinant factor VIII, the inhibitor and/or the anti-inhibitor according to any one of the preceding claims 1 to 9.

11. A diagnostic and/or purification device, which comprises at least one element which is selected from the group consisting of the epitope sequence, the pool of epitopes sequences, the complex, the inhibitor and/or the anti-inhibitor according to any one of the preceding Claims 1 to 9.

12. The device according to Claim 11, which is a diagnostic kit.

13. The device according to Claim 11, which is a chromatography column or filter.

14. Use of the pharmaceutical composition according to Claim 10 for the manufacture of a medicament in the treatment and/or the prevention of an immune disorder in a mammal induced by inhibitors of factor VIII polypeptide sequence.

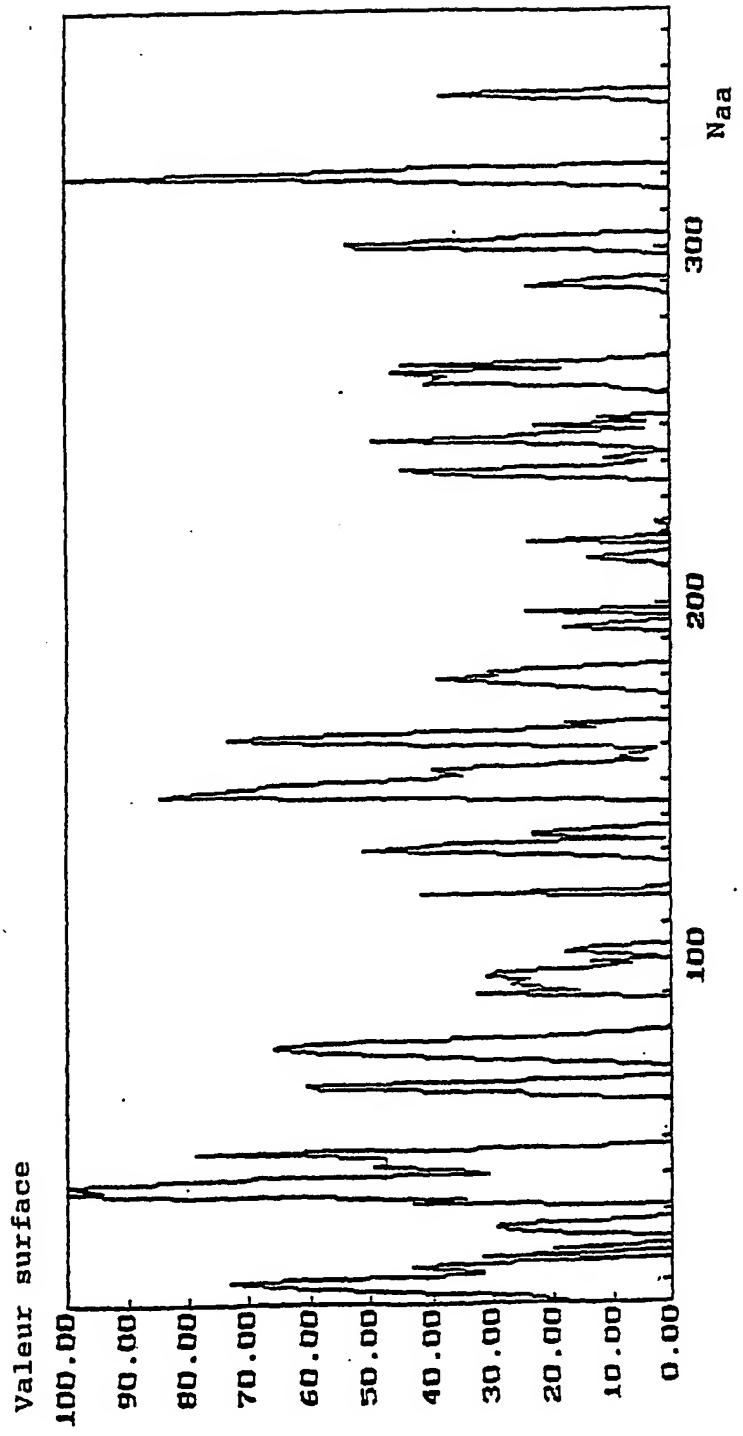
15. A method of treatment of a physiological fluid (serum), obtained from a mammal patient (including a human) wherein said physiological fluid is put into the chromatography column according to the claim 13 in order to allow a binding of the inhibitors of factor VIII polypeptide sequence present in said physiological fluid with epitope, pool or complex comprised in said chromatography column wherein after elution of the column a physiological fluid without inhibitors of factor VIII polypeptide sequence is recovered for its reinjection to the mammal patient.

16. A process for identifying and obtaining inhibitors and/or anti-inhibitors according to any of the preceeding Claims 6 to 9, comprising the steps of:

- selecting an element from the group consisting of the epitope sequence, the pool of epitopes sequences and/or the complex according to any one of the preceding claims 1 to 5, attached to a solid support (preferably a solid support of a chromatography column),
- passing a physiological fluid (serum) obtained from a patient containing inhibitors of factor VIII polypeptide sequence through said chromatography column,
- eluting said column, and
- collecting the fractions containing inhibitors of factor VIII polypeptide sequence which have exhibited an immunoaffinity with said element.

17. The process according to Claim 16, further comprising the steps of:

- attaching the collected inhibitors of factor VIII upon a solid support (preferably a solid support of a chromatography column),
- passing a physiological fluid from a mammal patient containing anti-inhibitors of factor VIII upon said solid support,
- washing the support, preferably by eluting said column, and
- collecting the fractions containing anti-inhibitors of factor VIII which have exhibited an immunoaffinity with said inhibitors of factor VIII.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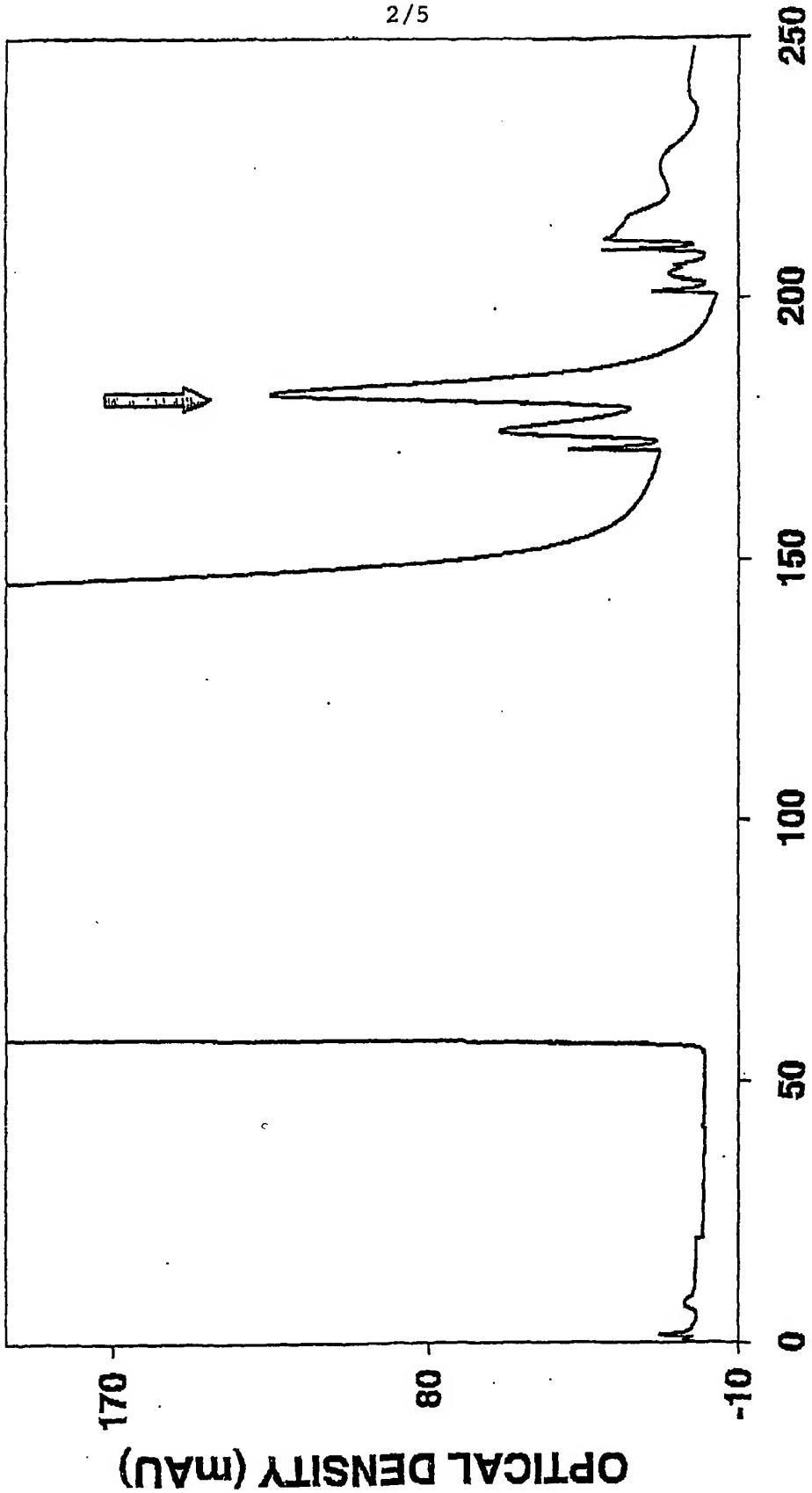
200
300
Naa

Fig: 1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150
200
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ELUTION VOLUME (ml)

Fig. 2a

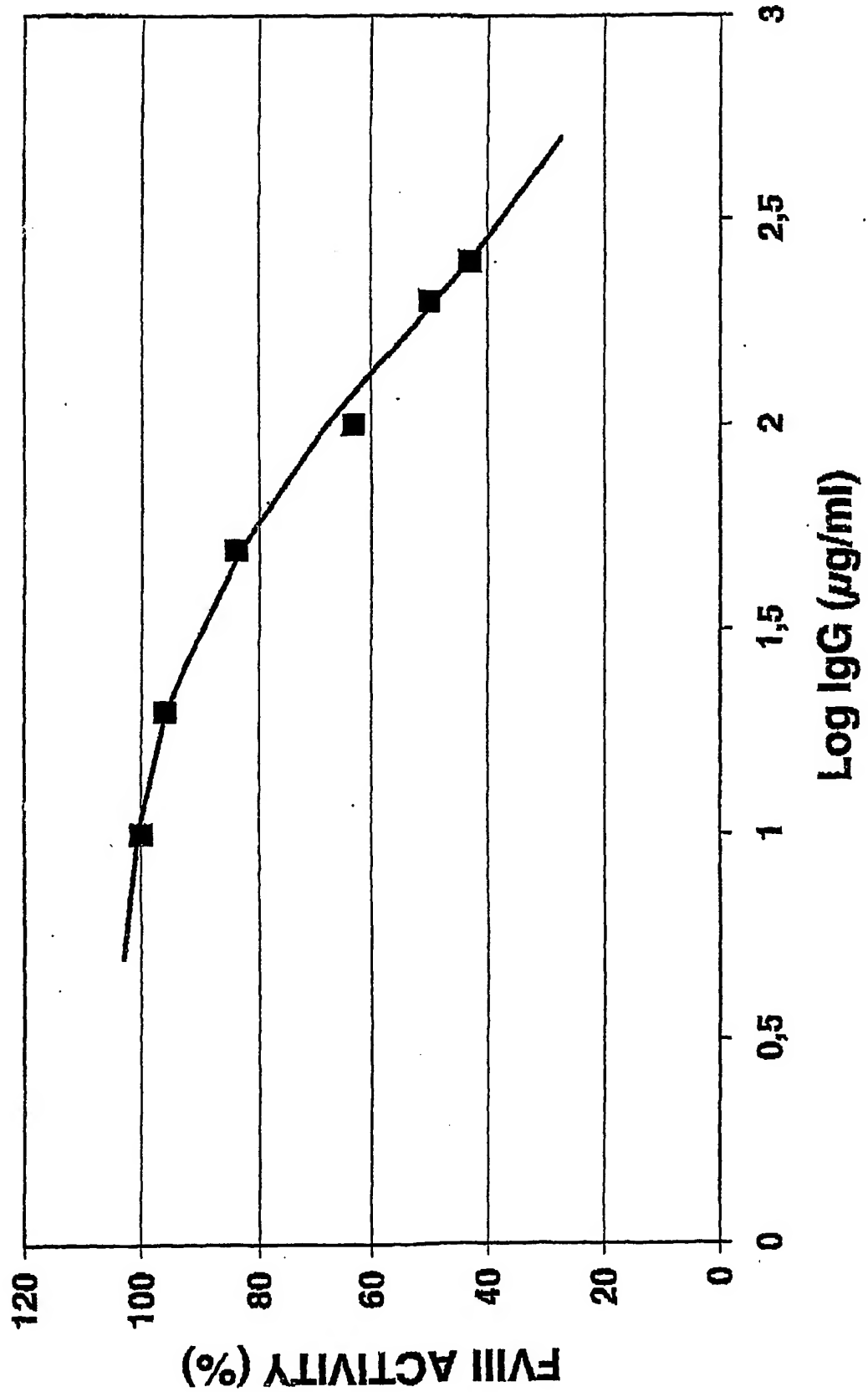
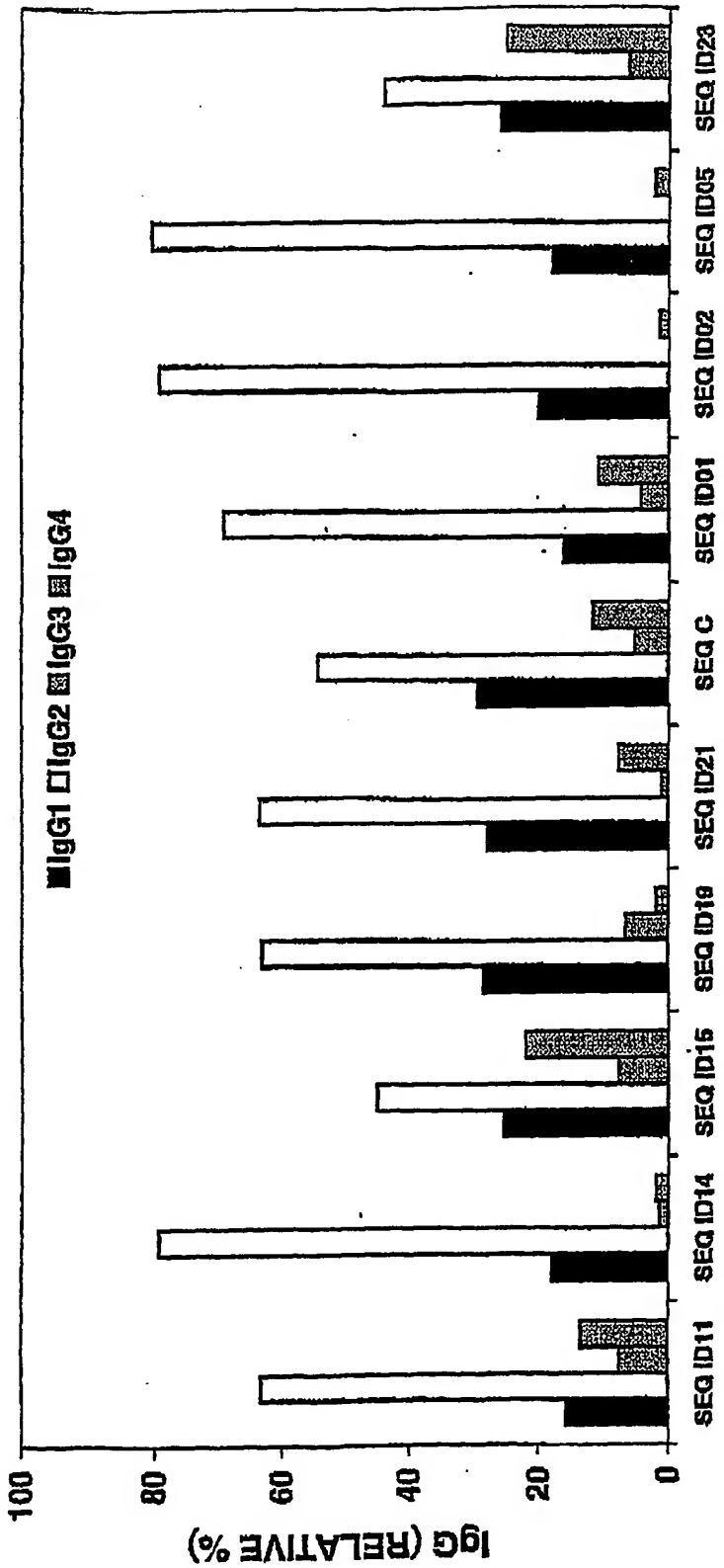


Fig. 2b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40
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SEQ ID15
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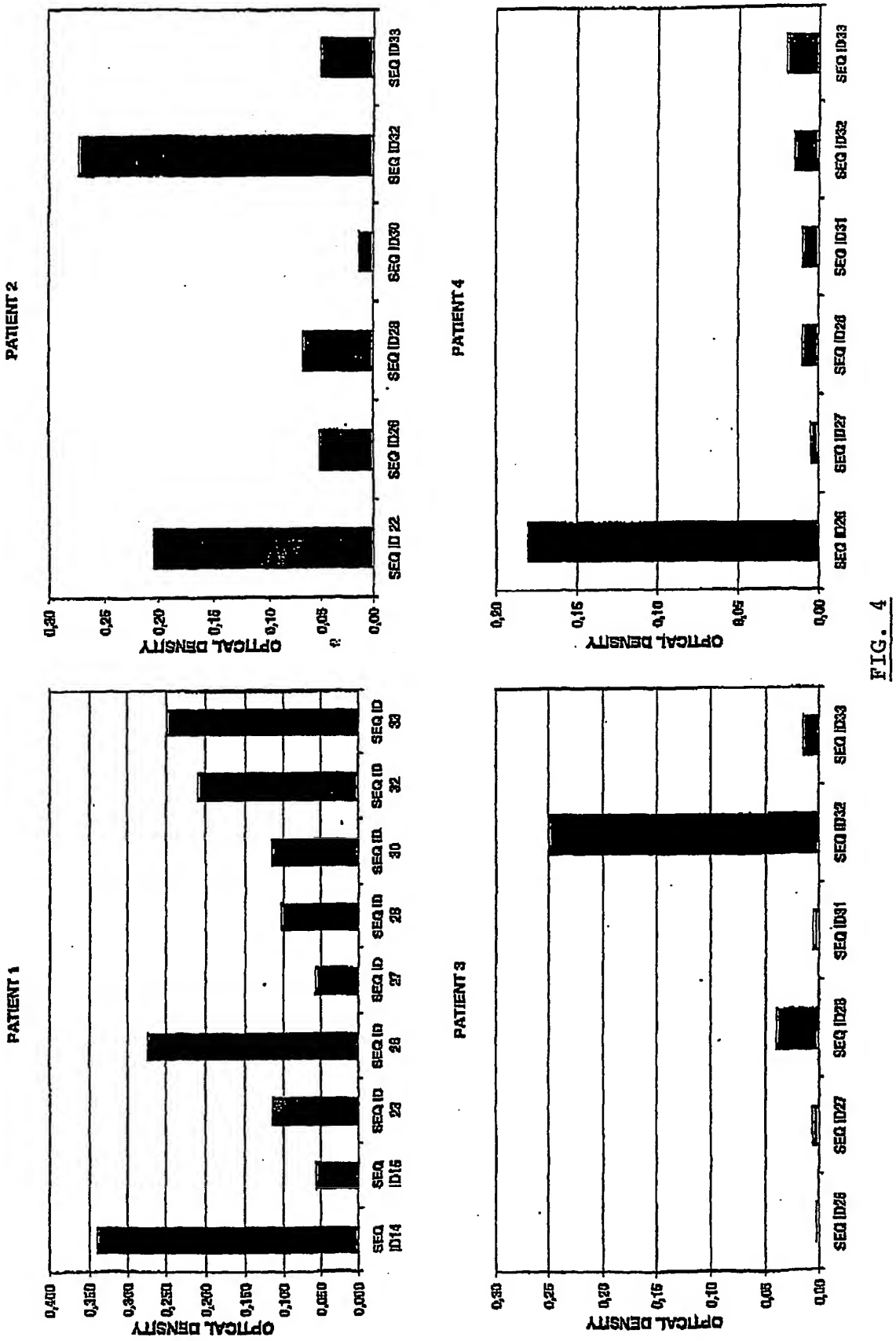
SEQ C
SEQ ID01
SEQ ID02
SEQ ID05
SEQ ID23

Fig. 3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SEQ ID 28
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SEQ ID 32
SEQ ID 33

FIG. 4

SEQUENCE LISTING

<110> Departement central defractionnement de la Croix-Rouge S.C.R.L.
Laub, Ruth
Di Gianbattista, Mario

<120> ANTIGENIC EPITOPES OF FACTOR VIII, INHIBITORS DIRECTED AGAINST SAID EPITOPES AND USE THEREOF

<130> P.CDFR.02B/WO.Ext

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